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Gene Polymorphisms of TNF-α-308(G/A), IL-10-1082(G/A), IL6-174(G/C) and IL1Ra(VNTR) in Egyptian Cases with type 1 Diabetes Mellitus

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Abstract

Background. Type 1 diabetes is a genetically conditioned autoimmune disease in which cytokines play an important role.

Objectives. To check for the association of polymorphisms of cytokine genes with the susceptibility and severity of type 1diabetes in cases from Egypt.

Subjects. This work included 50 cases with diabetes type 1 and 98 healthy individuals. The mean age of cases was 26 ± 7.93 years (range: 15-50 years). They included 20 males and 30 females. **Methods.** DNA was amplified using PCR-SSP for detection of polymorphisms related to TNF-α $^{-308}$ (G/A), IL-10 $^{-1082}$ (G/A), IL-6 $^{-174}$ (G/C) and IL-1Ra (VNTR).

Results. Total cases showed high significant frequency of IL-6^{-174} CC (P<0.05, OR=3.36) , IL-1Ra A_1A_1 (P<0.05, OR=3.86) and TNF-α^{-308} AA (OR=7.91, P<0.001) genotypes. These were considered risk genotypes for disease susceptibility. On the other hand, total cases showed low significant frequency with combined heterozygosity for IL-6^{-174} GC with IL-1Ra A_1A_2 (0.05) and TNF-α^{-308} GA with IL-10^{-1082} GA (P<0.001). These may be considered low risk genotypes.

Conclusions. Polymorphisms related to IL-6 , IL-1Ra ,IL-10 and TNF-α^{-308} genes may be considered genetic markers for type 1 diabetes among Egyptian cases. This may have potential impact on family counselling as well as future management plans.

Keywords : Type 1 diabetes , Cytokines , Gene polymorphism , IL-6 , IL-1Ra.

Abbreviations : IL; interleukin, IL-1Ra; IL-1 receptor antagonist, VNTR: variable number of tandem repeats, TNF; tumor necrosis factor, PCR-SSP: polymerase chain reaction with sequence specific primers. SNP: single nucleotide polymorphism, T1D: type 1 diabetes, DKA: diabetic ketoacidosis, IDDM : insulin dependent diabetes mellitus.

Introduction

Type 1 diabetes mellitus is an autoimmune disease in which the body destroys insulin-producing beta cells in the pancreas ***(Mathis et al., 2001).***

At least 20 different chromosomal regions have been linked to type 1 diabetes (T1D) susceptibility in humans, using genome screening, candidate gene testing, and studies of human homologues of mouse susceptibility genes ***(Pitkaniemi et al., 2007, Soria et al., 2007)***. The largest contribution from a single locus (IDDM1) comes from several genes located in the MHC complex (HLA alleles) on chromosome 6p21.3, accounting for at least 40% of the familial aggregation of this disease ***(Pociot and McDermott, 2002, Sia and Weinem, 2005).***

Cytokines also play an important role in type 1 diabetes as they are produced by immune cells and are thought to mediate beta cell destruction ***(Berg et al., 2006, Christen, 2007)***. Cytokines can cause beta cell death by direct cytotoxic effects, potentially acting through the induction of nitric oxide production. Alternatively, cytokines may enhance T cell-mediated beta cell destruction ***(Reddy and Young, 2002, Kainonen et al., 2006).***

The IL-1 cluster on human chromosome 2q12–2q14 harbors various promising candidate genes for inflammatory

diseases *(Ramadas et al., 2006)* . One of the important polymorp-hisms at the IL-1 gene cluster is that of IL-IRa(VNTR) caused by variable numbers of an 86 bp tandem repeat located in the second intron of the gene. The most common alleles have been termed allele one (A_1, 4 repeats), allele two (A_2, 2 repeats) in addition to other 3 alleles *(Tarlow et al., 1993)*.

Multiple immune mediators have been mentioned as playing a role in the pathomechanism of T1D. Interleukin IL-1beta, and tumor necrosis factor (TNF)-alpha play a central role in the autoimmune destruction of pancreatic beta-cells, whereas IL-6 inhibits TNF-alpha secretion, and may have some protective effects *(Gillespie et al., 2005)* . A polymorphism in the 5′ flanking region of the IL-6 gene on chromosome 7 at position -174 has been reported to exert an effect on its secretion and function*(Fishman et al., 1998)*.

Tumor necrosis factor alpha (TNF-α) is a potent inflammatory cytokine. In human, the TNF-α gene is located within the highly polymorphic major histocompatibility complex (MHC) region on chromosome 6p21.3 *(Wastowski et al., 2006)*. Many studies have shown that SNP at position –308 G/A and others within the TNF-α gene were associated with different inflammatory conditions *(Hajeer and Hutchinson, 2001)*.

IL-10 is an anti-inflammatory and immunosuppressive substance produced within the body and plays a role in the regulation of immune responses *(Tripp et al., 1993, Moore et al., 2001)*. The gene for IL-10 is in chromosome region 1q31-q32 *(Yu et al., 2004)*. The gene IL-10 has 3 biallelic single nucleotide polymorphisms (SNPs) within the promoter region, at positions -1082, -819, and -592 from the transcription initiation site. The -1082 (G/A) polymorphism was shown to affect the in vitro IL-10 production in peripheral blood cells from healthy individuals *(Crawley et al., 1999)*.

This work was planned in order to test the association of polymorphisms of 2 pro-inflammatory cytokines (TNF-α at position -308 and IL-6 at position -174) and 2 anti-inflammatory cytokines (IL-10 genes at position -1082 and of IL-1Ra VNTR) with type 1 diabetes in Egyptian cases.

Subjects and Methods

This study included 50 cases with type 1 diabetes recruited from the Department Of Endocrinology and Diabetes, Internal Medicine Specialist Hospital, Mansoura University, which is the main referral site in the Nile Delta Region of Egypt. They comprised 20 (40%) males and 30 (60%) females with an age ranging between 15-50 years and a mean ±SD of 26 ± 7.93 years. Of these cases, 21 (42%) had an age of onset of the disease ≤18 years, 33 (66%) were presenting with ketoacidosis, 13 (26%) had a positive parental consanguinity and 19 (38%) had a positive family history of type 1 diabetes.

Case genotypes were compared to 98 healthy unrelated volunteers from the same locality. They included 52 males and 46 females with a mean age ± SD of 44.9±6.7 years. Informed consent was taken from all subjects and an approval was obtained from University Scientific and Ethical Committees to carry out the research as well.

DNA extraction and purification

After obtaining informed consent from all cases and controls, venous blood samples (3 ml) were collected on EDTA (ethylenediamine tetra acetate) containing tubes, DNA was extracted promptly using DNA extraction and purification kit (Gentra Systems, USA) according to manufacturer's instructions and then stored at -20 ^{0} C till use.

PCR amplification

Three single nucleotide polymorphisms (SNPs) were analyzed including promoter sites TNF-α $^{-308}$ (G/A), IL-10 $^{-1082}$ (G/A) and IL-6 $^{-174}$ (G/C) as well as IL-1Ra ^{VNTR} as previously described (Sargen *et al.*,2000; Cavet *et al.*,2001; Cavet *et al.*,1999; Wilkinson *et al.*,1999). For TNF-α, IL-6 and IL-10 SNPs identification, PCR with sequence-specific primers (PCR-SSP) in two reactions employing a common forward and 2 reverse primers was used, and for IL-1Ra VNTR polymorphism, a single PCR reaction employing a forward and a reverse primers was used(All primers, Taq polymerase, dNTP, and $MgCl_2$ were purchased from QiaGene (QiaGene, USA)). The assay was performed in Techne-Genius

thermal-cycler (England). Briefly, 100-500 ng of genomic DNA was added to 25 µl of reaction mixture containing 1 µM of each common/specific primer, 200 µM of each dNTP, and 1 U of Taq DNA polymerase. We were careful to have master mixes for multiple cases and also for different polymorphisms at the same sitting with confirmation of the negative amplification to obtain accurate subject genotyping.

Detection of amplified products

The entire reaction volume plus 5 µl of bromophenol blue track dye were loaded into 2% agarose gel (Boehringer Mannheim) containing ethidium bromide. Gels were electrophoresed for 20 minutes at 200 V, photographed under UV light (320 nm) and then scored for the presence or absence of an allele specific band.

Statistical analysis: Data were processed and analyzed using the Statistical Package of Social Science (SPSS, version 10.0). The frequency of studied allelic polymorphisms among cases was compared to that of controls describing number and percent of each and tested for positive association using Fisher's exact test (modified Chi square test) and Odds ratio with a minimum level of significance of <0.05.

Results

Analysis of TNF-α^{-308} polymorphism (table 1,2, fig.1), showed that allele A is significantly higher in total cases compared to controls (OR=2.0, P<0.05). The same was observed for AA genotype (OR=7.91, P<0.001). On the other hand, allele G has shown significant lower frequency among total cases (OR=0.5, P<0.05) . The same was observed with GA genotype (OR=0.12 , P<0.001).

Analysis of IL-10^{-1082} (G/A) polymorphism (table1,2, fig.2), showed that there was no significant difference in frequencies of all genotypes and alleles in total cases as well as between different studied subgroups.

Analysis of IL-6^{-17}CC polymorphism (table 1,2, fig.3), showed that IL-6^{-17}CC genotype is significantly higher in total cases compared to controls (OR=3.36, P<0.05). Interestingly, no significant difference was found in the frequencies of all studied alleles.

Analysis of IL-1Ra VNTR polymorphism (table 1,2, fig.4), showed that homozygous form A_1/A_1 was found significantly high in total cases ((P<0.05, OR=3.68, 95%CI= 1.56-8.68) as well as cases subgroups, while the heterozygous form A_1/A_2 was found significantly low among total cases (OR=0.27,P<0.05) .

Analysis of the frequency of combined phenotypes (table 4), showed that the combined genotypes [TNF-α^{-308} A/A with IL-10^{-1082} G/G] was of highest significant frequency among cases (OR=14.5, P<0.05) and [IL-6^{-174} (C/C) with IL-1Ra (A_1/A_1)] (OR= 3.8, P<0.05). On the other hand, maximum significant lower frequency was found among cases with combined heterozygosity genotypes of [IL-10^{-1082} (G/A) with TNF-α^{-308} (G/A)] (OR=0.15, P<0.001).

Table 1. IL-6, IL-10 , TNF-α and IL-1Ra genotype frequencies among type 1 diabetes cases compared to controls with their statistical significance

	Cases n=50(%)	Controls n=98(%)	OR(95%CI)
IL-6 Genotypes			
GG	3(6.0)	5(5.1)	1.19(0.27-5.2)
GC	38(76.0)	87(88.8)	0.40(0.16-0.98)
CC	9(18.0)*	6(6.1)	3.36(1.12-10.1)
IL-10 Genotypes			
GG	5(10.0)	5(5.1)	2.17(0.67-7.51)
GA	40(80.0)	85(86.7)	0.61(0.24-1.51)
AA	5(10.0)	8(8.2)	1.25 (0.38-4.04)
TNF-α Genotypes			
GG	6(12.0)	6(6.1)	2.1(0.6-6.7)
GA	19(38.0)**	81(82.7)	0.1(0.1-0.3)
AA	25(50.0)**	11(11.2)	7.9(3.4-18.3)
IL-1Ra genotypes			
A1A1	42(84.0)*	57(58.2)	3.68(1.56-8.68)
A1A2	8(16.0)*	40(41.80)	0.27(0.11-0.63)

*P<0.05 **P<0.001 OR(95th CI)= Odds Ratio (95th Confidence Interval)

Table 2. IL-6, IL-10 , TNF-α and IL-1Ra allele frequencies among type 1 diabetes cases compared to controls with their statistical significance

	Cases n=50(%)	Controls n=98(%)	OR(95%CI)
IL-6 Alleles			
G	44(44.0)	97(49.5)	0.80(0.59-1.30)
C	56(56.0)	99(50.5)	1.24(0.87-2.02)
IL-10 Alleles			
G	50(50.0)	95(48.5)	1.16(0.65-1.72)
A	50(50.0)	101(51.5)	0.94(0.58-1.52)
TNF-α alleles			
G	31(31.0)*	93(47.4)	0.5(0.3-0.8)
A	69(69.0)*	103(52.6)	2.0(1.2-3.3)
IL-1Ra alleles			
A1	92(92.0)*	154(79.4)	2.98(1.33-6.66)
A2	8(8.0)*	40(20.6)	0.33(0.15-0.74)

*P<0.05 = significant , OR(95% CI)= Odds Ratio (95% Confidence Interval)

Table 3. Frequency of combined genotypes of studied cytokine gene polymorphisms among cases compared to controls with their statistical significance

	Cases n=50(100%)	Control n=98(100%)	OR(95%CI)
TNF-α-308A/A and IL-10-1082G/G	3(6.0)*	0(0.0)	14.5(1.03-286.9)
TNF-α-308A/A and IL-10-1082G/A	19(38.0)**	8(8.2)	6.89(2.7-17.3)
TNF-α-308G/A and IL-10-1082G/A	15(30.0)**	73(74.6)	0.15(0.7-0.3)
TNF-α-308G /A and IL-6-174G/C	18(36.0)**	72(73.5)	0.2(0.09-0.4)
IL-6-174CC and IL-1Ra A1A1	7(14.0)*	4(4.1)	3.8(1.2-13.8)
IL-6-174GC and IL-1Ra A1A2	5(10.0)*	34(35.0)	0.2(0.2-0.6)
IL-6-174C/C and TNF-α-308A/A	5(10.0) *	2(2.04)	5.3 (1.09-28.6)
IL-1Ra A1A1 and IL-10-1082A/A	5(10.0)*	2(2.04)	5.3(0.9-28.5)
IL-1Ra A1A2 and IL-10-1082G/A	8(16.0)*	32(32.6)	0.4(0.2-0.9)
IL-1Ra A1A2and TNF-α-308G/A	1(2.0)**	32(32.6)	0.04(0.01-0.3)
IL-1Ra A1A1and TNF-α-308A/A	22(44.0)**	5(5.1)	14.6(5.1-42.1)
IL-10-1082G/A and IL-6-174G/C	30(60.0)*	76(77.6)	0.4(0.2-0.9)

*P<0.05 **P<0.001 OR(95% CI)= Odds Ratio (95% Confidence Interval)

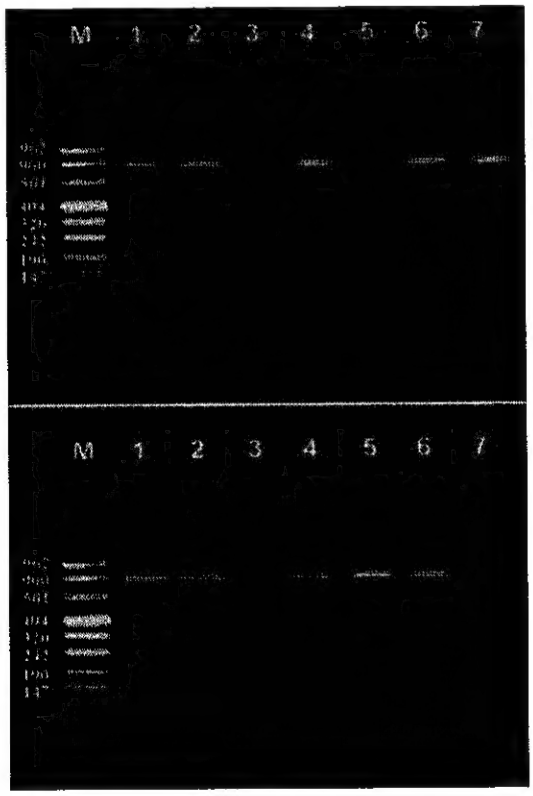

Fig. (1): PCR amplification for TNF-α at position -308 polymorphisms using SSP for G allele (above) and A allele (below) (band size: 836 bp). showing: lane M: DNA size marker, lanes 1,2,4,6 (+ve for G and A alleles giving G/A genotype), lane 5 (-ve for G allele and + ve for A allele giving A/A genotype), lane 7 (+ ve for G allele and – ve for A allele giving G/G genotype) and lane 3 –ve control.

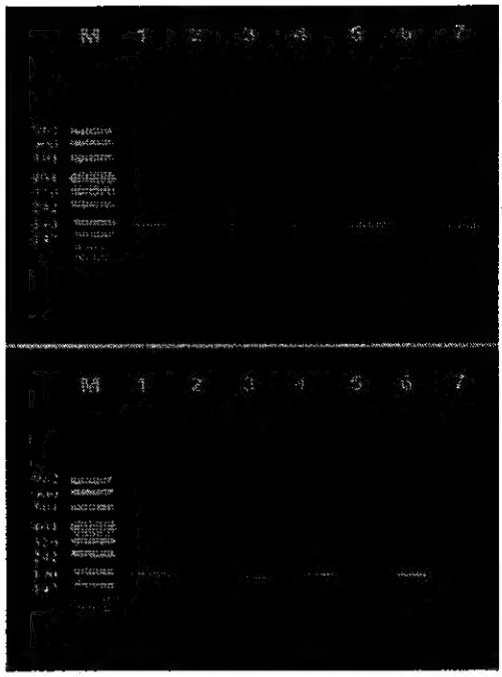

Figure 2. PCR amplification for IL-10 at position -1082 polymorphisms using SSP for G allele (above) and A allele (below) (band size 179bp) showing: lane M: DNA size marker, lanes 1,3,4 (+ve for G and A alleles giving G/A genotype), lane 6 (– ve for G allele and + ve for A allele giving A/A genotype), lanes 5,7 (+ ve for G allele and – ve for A allele giving G/G genotype) and lane 2 – ve control.

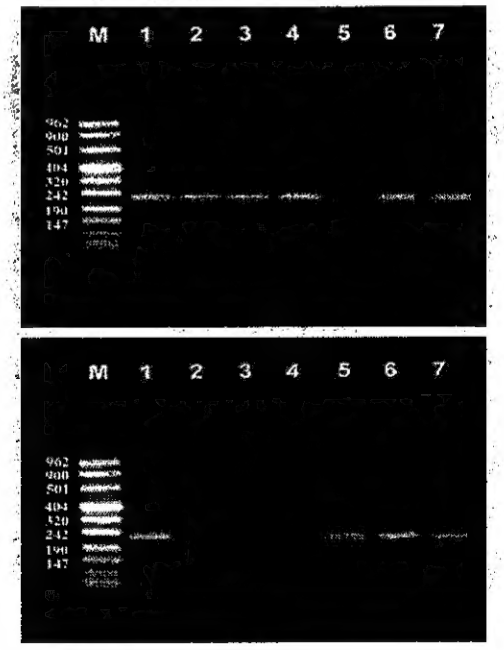

Figure 3. PCR amplification for IL-6 at position -174 (G/C) polymorphisms using SSP for G allele above and C allele below (band size 234 bp) show: lane M: DNA size marker, lanes 1,6,7 (+ve for G and C alleles giving G/C genotype), lanes 2,3,4 (+ve for G allele and –ve for C allele giving G/G genotype) and lane 5 (-ve for G allele and + ve for C allele giving C/C genotype).

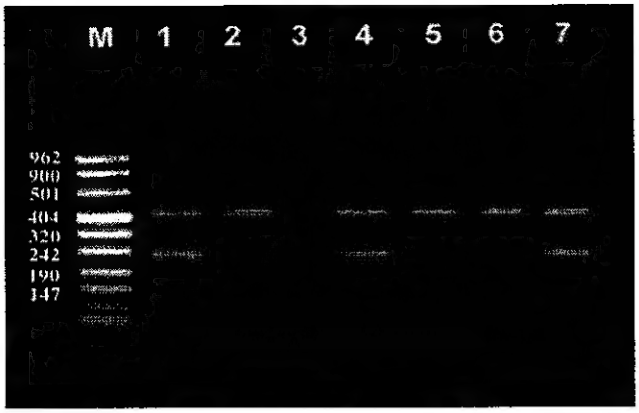

Figure 4. PCR amplification for IL-1Ra (VNTR) in intron 2 showing two alleles : band size 410 bp for A_1 allele and band size 240 bp for A_2 allele. Lane M shows DNA size marker. Lanes 1,4,7 show +ve bands of A_1 and A_2 alleles giving A_1A_2 genotype, lanes 2,5,6 show only one band of A_1 allele giving A_1A_1 genotype while lane 3 is a negative control sample with no DNA showing failure of amplification.

Discussion

The presence of inherited immunoregulatory dysfunction including the possibility of stimulation of certain cytokines resulting in a specific clinical behavior is well reported in T1D. Cytokines largely influence the immunologic function, initiation and progression of the disease ***(Greenfield et al., 1998).***

Analyzing studied Egyptian cases for combined genotypes, a certain pattern could be found to play a role in type 1 diabetes disease susceptibility and/or severity. This included IL-6^{-174} CC with IL-1Ra A_1A_1, that were shown to be significantly higher among all cases. On the other hand, heterozygous genotypes IL-6^{-174}GC with IL-1Ra A_1A_2 were found significantly low among cases pointing to possible protective effect of these genotypes.

This result was in agreement to what was previously reported in Hungary that T1DM was significantly associated with IL-6^{-174}CC genotype; (p <0.01, 95% CI: 1.2-7.1) ***(Martha et al., 2004; Hermann et al., 2005)*** . Likewise, English researcher found that the IL6-174CC genotype was significantly less frequent in females diagnosed after than in those diagnosed before the age of 10 years (19 vs. 13%, P = 0.016). No genotype difference was observed in males stratified for age at onset ***(Gillespie et al., 2005)*** . However, in another study among Caucasoid patients with type 1 diabetes, homozygous GG genotype was found increased in the patients compared to the normal controls (50.6% vs. 33.3%, p < 0.002) ***,(Jahromi et al., 2000).***

Polymorphisms in the IL-1 receptor genes were found to have an association with T1D among Dalmatian population ***(Zemunik et al., 2005).*** On the other hand, Danish authors reported no association of IL-Ra polymorphisms with T1D ***(Kristiansen et al., 2000)*** contrasting what was reported before in the same population of increased frequency of the A1A1 genotype with IDDM P=0.025) ***(Mandrup-Poulsen et al., 1994; Pociot et al., 1994; Mandrup-Poulsen, 1996).***

In the current study, total cases showed a significant higher frequency of TNF-α^{-308} AA genotype. Thus, TNF-α^{-308} A allele and AA genotype may be considered a risk markers for type 1 diabetes among Egyptian families. On the other hand, significant lower frequency was observed with heterozygous genotype GA that was considered a low risk or protective genotype. The same was previously reported by ***Kumar et al.,(2007)*** that TNF-α^{-308} AA genotype was significantly increased in North Indian patients with T1D compared with the controls . Also, in a study among Polish subjects, authors noted a significant difference of cytokine

synthesis levels and cytokine genotype distribution among T1D cases compared to controls. Of these cytokines, TNF-α^{-308} GA genotype was found only in cases but not in controls ***(Siekiera et al., 2002).*** On the other hand, ***Deng et al.,(1996)*** reported that no primary association was found between the - 308 polymorphism in TNF-alpha promoter region and T1D cases from two ethnic populations; U.S. Caucasians and Chinese in Taiwan .

Regarding interleukin-10 gene polymorphism at position -1082, we have found different in significant for genotype and alleles among cases of the disease. Such polymorphism is known to be associated with the reduction of secreted IL-10 which may support the concept of accelerated Th1 reactivity in these cases. These results are different with that reported by Polish authors who demonstrated that IL-10-1082 polymorphism is related to T1D especially AA genotypes ***(Siekiera et al., 2002).*** In another French study, ***Reynier et al.,(2006)*** reported that the IL-10^{-1082} polymorphism was significantly associated with GAD and IA-2 antibodies at clinical onset of T1D. They added that IL-10 promoter gene variants may contribute, but to a minor extent, to disease susceptibility in juvenile type 1 diabetes and should not be included in the routine genetic screening of high-risk individuals ***(Reynier et al., 2006).*** Also, ***Ide et al.,(2003)*** in a study on Japanese cases demonstrated that IL-10 gene promoter region polymorphisms are not associated with genetic susceptibility to type 1 diabetes group. In a previous study by the same group in Japan, ***Ide et al.,(2002)*** have reported that only ages older than 18 years showed significant higher frequency of AA genotype. In addition, a minor role was assumed to IL-10 genotypes in the autoimmune diabetes risk in Spanish type 1 diabetes patients ***(Urcelay et al., 2004).***

Conclusions

Controversies may be explained by the assumption that these genotypes are population specific and co-segregate with the disease genes in different forms among different ethnic groups. Based on this study, we can conclude that cytokine gene polymorphisms related to IL-6,IL-1Ra ,TNF-a and IL-10 genes may be considered as genetic markers for type 1 diabetes among Egyptian cases. We recommend undertaking more family studies with linkage analysis to evaluate the actual risk among families with multiplex cases.

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الطراز المظهري لجين السيتوكينز في حالات مرضى السكري من النوع الأول في مصر

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استهدفت الدراسة موضوعا حيويا وهاما فى المجال الفسيولوجي الجزيئي الطبي وكان من الجينات التي تستحق الدراسة وتعتبر مهمة فى المرض السكري من النوع الأول جينات الوسائط الخلوية (السيتوكينات) التي تعمل في بداية الإلتهاب مثل الانترلوكين-6 ومعامل موت الخلايا المبكر -الفا وجينات الوسائط الخلوية (السيتوكينات) مضادة للإلتهاب مثل الانترلوكين-10 والانترلوكين-1 مضاد المستقبل و تشكل الوسائط الخلوية (السيتوكينات) مجموعة صغيرة من الجزيئات الذائبة أو الخلايا المرتبط سطحها بالبروتين التى تنقل المعلومات من خلية إلى أخرى.

أوضحت هذه الدراسة أن هناك أشكال معينة في التركيب الجينى الوراثي للسيتوكينات موجودة بنسبه إحصائية معنوية فى الحالات المرضية عنها في الأصحاء. اشتملت هذه التركيبات الجينية على التركيب الجينى المتجانس وراثياً للانترلوكين -6 (عند -174) سيتوزين/سيتوزين ومعامل موت الخلايا المبكر-ألفا (عند -308) أدنين / أدنين والانترلوكين-1 مضاد المستقبل VNTR جين1/ جين1 و التي يمكن أن تعتبر خطيرة وتعرض الأشخاص حامليها لتطور المرض.

أيضا أوضحت هذه الدراسة أن هناك أشكال معينة فى التركيب الجيني للسيتوكينات موجودة بنسبة إحصائية معنوية منخفضة في الحالات المرضية عنها في الأصحاء. اشتملت هذه التركيبات الجينية على التركيب الجينى الغير متجانس وراثياً لمعامل موت الخلايا المبكر-الفا(عند -308) جوانين/أدنين والانترلوكين-1 مضاد المستقبل (VNTR) جين1/جين2 و التي يمكن أن تعتبر حماية ضد تطور المرض. بالإضافة إلي ذلك فان استخدام تقنية (SSP- PCR) تعتبر مهمة للتعرف على المرض وبيان شدة خطورته.